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(54) **Title**: LIPOSOME FORMULATION HAVING HYDROPHILIC AND HYDROPHOBIC PHARMACEUTICAL COMPOUNDS CO-ENCAPSULATED THEREIN

(57) **Abstract**: The present invention features liposomal formulations composed of at least one hydrophilic pharmaceutical compound loaded in the aqueous core of the liposome and at least one hydrophobic pharmaceutical compound in the bilayer of the liposome; and a method for producing the same. In so far as the pharmaceutical compounds are chemotherapeutic agents, the present invention also embraces a method for preventing the development of chemotherapeutic drug resistance.

# LIPOSOME FORMULATION HAVING HYDROPHILIC AND HYDROPHOBIC PHARMACEUTICAL COMPOUNDS CO-ENCAPSULATED THEREIN

## Background of the Invention

Liposomes are spherical vesicles of self-closed hydrated bilayers of amphiphilic lipids surrounding a generally central inner aqueous phase core which can differ in composition from the extraliposomal aqueous medium (Bangham & Horne (1964) *J. Mol. Biol.* 8:660-668). The lipid chains may be liquid-crystalline or solid-like gel phases. Liposomes are colloidal particles ranging in diameter from 20 nm to 5000 nm. Depending on the size and the number of constituent lamellar layers, these are classified as small or large unilamellar vesicles, and as multilamellar vesicles. The multilamellar vesicles have additional water layers trapped adjacent to the hydrophilic ends (polar head groups) between the regular dual arrays of the lipophilic (hydrophobic) alkyl chains (fatty tails).

The lipid bilayer of the unilamellar vesicles is akin in composition and structure to the outer membrane of eukaryotic cells. The vesicle bilayer provides a significant controllable barrier to the movement of various molecules and ions between the inner aqueous core and the bulk aqueous phase surrounding the liposome (Bangham, et al. (1965) *J. Mol. Biol.* 13:238-252). This barrier function is paramount in many applications including drug delivery vehicles.

Liposomes are conventionally prepared from natural phospholipids and synthetic analogues such as the electrical charge neutral zwitterionic phosphatidylcholines. Minor proportions of anionic phospholipids, such as phosphatidylglycerols, can be added

to generate a net negative surface charge for colloid stabilization.

Natural compounds (Li, et al. (2005) *Cancer Res.* 104(6):1322-1331; U.S. Patent Application No. 20060067998) as well as synthetic compounds have been loaded into liposomal bilayers (Perez-Soler, et al. (1992) Board of Regents, The University of Texas System; Perez-Soler, et al. (1994) *Cancer Chemother. Pharmacol.* 33(5):378-384). Furthermore, combinations of synthetic compounds (*e.g.*, doxorubicin and vincristine) have been encapsulated together in the aqueous core of liposomes (Abraham, et al. (2004) *Clin. Cancer Res.* 10::728-738). Moreover, amphiphilic compounds have been encapsulated in liposomes. See U.S. Patent No. 7,368,129.

**Summary of the Invention**

The present invention is a liposome formulation having at least one hydrophilic pharmaceutical compound loaded in the aqueous core of the liposome and at least one hydrophobic pharmaceutical compound in the bilayer of the liposome. In some embodiments, the hydrophilic pharmaceutical compound is a synthetic compound and the hydrophobic pharmaceutical compound is a natural compound. In other embodiments, the hydrophilic pharmaceutical compound is a natural compound and the hydrophobic pharmaceutical compound is a synthetic compound. In still further embodiments, the hydrophilic pharmaceutical compound and the hydrophobic pharmaceutical compound are natural compounds. In particular embodiments, the hydrophilic pharmaceutical compound and the hydrophobic pharmaceutical compound are chemotherapeutic agents.

A method for preventing the development of chemotherapeutic drug resistance and a method for producing a multi-drug-loaded liposome are also provided.

5 **Brief Description of the Drawings**

Figure 1 shows the *in vitro* cytotoxicity of drug-loaded liposomes.

Figure 2 shows the inhibition of cisplatin-resistance development in SCC9 cells upon incubation with multi-drug 10 liposomes. * Indicates that the differences were statistically significant.

Figure 3 shows cell survival profiles at 7 and 15 days after incubation with the three different treatment groups indicated. Results of analyses with starting doses of 500 15 nM and 1 $\mu$M are shown in Figures 3A and 3B, respectively.

**Detailed Description of the Invention**

The present invention features the encapsulation of one or more hydrophilic compounds with one or more 20 hydrophobic compounds into a single drug carrier. In accordance with the present invention, at least one hydrophilic compound is encapsulated in the aqueous core and one hydrophobic compound in the bilayer of a liposome. By way of illustration, it was found that by loading 25 cisplatin (a synthetic hydrophophilic compound) and curcumin (a natural hydrophobic compound) together in a liposome, enhanced suppression of tumor cell proliferation was achieved. Indeed, the delivery of curcumin along with cisplatin was found to make cancer cells more sensitive to 30 the effects of cisplatin thereby achieving increased cell cytotoxicity and reduced development of drug resistance. Thus, the invention embraces co-encapsulating at least one hydrophilic pharmaceutical compound into the aqueous core

of a liposome of any size (*e.g.*, 50 nm - 10,000 nm) and at least one hydrophobic pharmaceutical compound into the lipid bilayer to provide a multi-drug-loaded liposome. This invention finds application in the delivery of a variety of pharmaceutical compounds for the prevention or treatment of disease or for cosmetic applications.

Liposomes of the present invention can be prepared from any vesicle-forming lipid. A "vesicle-forming lipid" refers to a lipid, which has hydrophobic and polar head group moieties, wherein said lipid can form bilayer vesicles in water, as exemplified by phospholipids, or can be stably incorporated into lipid bilayers, with the hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and the polar head group moiety oriented toward the exterior, polar surface of the membrane. The vesicle-forming lipids of this type typically include one or two hydrophobic acyl hydrocarbon chains or a steroid group, and can contain a chemically reactive group, such as an amine, acid, ester, aldehyde or alcohol, at the polar head group. Included in this class are phospholipids where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. Representative examples are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidic acid (PA), phosphatidyl inositol (PI), sphingomyelin (SM), negatively charged lipids such as dimyristoyl phosphatidyl glycerol (DMPG), and positively charged lipids such as 1,2-distearoyl-3-trimethylammonium propane (DSTAP). The liposomes can also contain sterols, such as cholesterol, which do not form liposomes themselves but can be incorporated into, and may stabilize, liposomes containing lipids such as those described above.

Various vesicle-forming lipids, as defined above, can be used in the present liposomal compositions, according to methods well-known in the art. Preferred lipids for the current invention allow long-term storage of the liposome-entrapped compounds and effective release of these compounds upon administration. Representative lipids include, but are not limited to, dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), cholesterol, egg phosphatidylcholine (egg PC), phosphatidyl ethanolamine (PE), distearoyl phosphatidyl ethanol-amine (DSPE), phosphatidyl inositol (PI), 1,2-distearoyl-3-trimethylammonium propane (DSTAP), 1,2-dimyristoyl-3-trimethylammonium propane (DMTAP), and combinations thereof. In so far as combinations of lipids are generally most effective (see, *e.g.*, Kedar, et al. (1994) *J. Immunotherapy* 16:47-59), the present invention particularly embraces the use of a combination of two or more of the above referenced lipids. In certain embodiments, the instant liposomes are composed of DMPC and DMPG. In particular embodiments, the instant liposomes are composed of DMPC and DMPG in a molar ratio of 1:1.

Liposomes can be prepared using the methods disclosed herein or by a variety of techniques, such as those detailed in U.S. Patent No. 4,235,871; Liposome Technology (1984) CFC Press, NY; Liposomes by Ostro (1987) Marcel Dekker; Lichtenberg & Barenholz (1988) *Methods Biochem. Anal.* 33:337-462; and U.S. Patent No. 5,283,185. For example, to form multilamellar vesicles (MLVs, *i.e.*, vesicles having a mean diameter of approximately 250 to 2000 nm), a mixture of vesicle-forming lipids dissolved in a suitable solvent is evaporated in a vessel to form a thin film, which is then hydrated by an aqueous medium to form

MLVs, typically with sizes between about 0.1 to 10 microns. MLVs can then be reduced to a desired size range by extruding the aqueous suspension through a polycarbonate membrane having a selected uniform pore size, typically 5 0.05 to 1.0 microns.

Preparations of MLVs or REVs (described below) may be treated, *e.g.*, by extrusion, sonication or high pressure homogenization, to produce unilamellar vesicles. Small unilamellar vesicles (SUVs) are characterized by sizes in 10 the 20 to 100 nm range, while large unilamellar vesicles (LUVs) are defined as those having mean diameters of about 100 to 200 nm. SUVs can also be formed directly by high pressure homogenization of an aqueous dispersion of lipids.

In the reverse phase evaporation method (Szoka, et al. 15 (1980) *Ann. Rev. Biophys. Bioeng.* 9:467), a nonaqueous solution of vesicle-forming lipids is dispersed with a smaller volume of an aqueous medium to form a water-in-oil emulsion. Agents to be incorporated are included in the lipid solution, in the case of the hydrophobic compound, or 20 in the aqueous medium, in the case of the hydrophilic compound. After removal of the lipid solvent, the resulting gel is converted to liposomes. These reverse phase evaporation vesicles (REVs) have typical average sizes between about 0.2 to 4 microns and are predominantly 25 oligolamellar, that is, containing one or a few lipid bilayer shells. The REVs can be sized by extrusion, if desired, to give oligolamellar vesicles having a maximum selected size between about 0.05 to 1.5 microns.

Regardless of the specific methodology and size of 30 liposome achieved, multi-drug-loaded liposomes of the present invention are generally produced by combining

(a) a lipid and hydrophobic pharmaceutical compound mixture with

(b) an aqueous phase containing a hydrophilic pharmaceutical compound

so that the hydrophobic pharmaceutical compound is incorporated or intercalated into the lipid bilayer of the
5 liposome and the hydrophilic pharmaceutical compound is trapped in the aqueous core of the liposome.

Liposome compositions of the invention may be treated after final sizing, if necessary, to remove free (non-entrapped or -encapsulated) agent. Conventional separation
10 techniques, such as centrifugation, diafiltration, and molecular-sieve chromatography are suitable for this purpose. The composition can also be sterilized by filtration through a conventional 0.22 or 0.45 micron depth filter.

15 In addition to lipids and the active compounds, the instant liposomes can also include other components. For example, stabilizers can also be added to the liposomal compositions. For example, addition of a metal chelator such as DESFERAL or diethylenetriamine pentaacetic acid
20 (DTPA) to the lyophilization medium, at a concentration of 100 $\mu$M, has been shown to reduce activity loss of entrapped active compounds during liposome preparation and storage at 4°C.

The stability of the liposomes of the present
25 invention can be measured by specific assays to determine the physical stability and biological activity of the liposomes over time in storage. The physical stability of the liposomes can be measured as disclosed herein or any other suitable method for determining the diameter,
30 including for example, gel filtration chromatography or by means of quasi-elastic light scattering.

For the purposes of the present invention, a pharmaceutical compound is a compound that provides a

therapeutic benefit (*e.g.*, prevention or treatment of a disease) or beneficial biological activity (*e.g.*, non-medicinal uses such as vitamin supplementation). In this regard, the pharmaceutical compounds of the invention are encapsulated in a liposome of the present invention as more than a simple additive or carrier. Rather, the pharmaceutical compounds are selected to achieve a therapeutic benefit or beneficial biological activity, with preference for pharmaceutical compounds that enhance the activity of one or more of the compounds co-encapsulated, and/or provide synergistic or complementary effects.

Pharmaceutical compounds of the present invention include compounds selected for medical use such as chemotherapeutic agents or cytotoxins; agents to combat infectious organisms, such as antibiotics, anti-virals and fungicides; immunological components, such as antibodies or fragments thereof, antigens, cytokines and anti-inflammatory agents in general; proteins such as enzymes, hormones, and neurotransmitters; anesthetics; blood components such as hemoglobin and coagulants; and a variety of nucleic acid molecules, constructs or vectors, including those that express any of the foregoing components and those that include antisense nucleic acids and ribozymes. In other embodiments, a pharmaceutical compound can be a nutritional supplement, pheromone or contrast agent. Cosmetics are also embraced by the present invention.

A hydrophilic pharmaceutical compound is a pharmaceutical compound with water solubility greater than about 100 μg/ml. In accordance with the present invention, a hydrophilic pharmaceutical compound is soluble in the aqueous core of the liposome. Hydrophilic pharmaceutical compounds can be macromolecules such as peptides, proteins, peptidomimetics, polysaccharides cytokines, nucleotides,

nucleosides, genetic materials, toxoids, serum vaccines or combinations thereof. Examples of hydrophilic pharmaceutical compounds include, but are not limited to, chemotherapeutic agents such as cisplatin, gemcitabine, 5 doxorubicin, daunomycin, methotrexate, imatinib mesylate, or mitomycin; antiinflammatory agents such as acetaminophen; enzymes such as L-asparaginase; steroids such as prednisolone or hydrocortisone; antihypertensive agents such as captopril or minoxidil; antibiotics such as 10 tetracycline, chloramphenicol, and rifamycin; interleukins such as IL-12 or IL-9; interferons such as IFNγ, IFN-α, or IFN-β; haemoglobin; antibodies; nucleic acid molecules; and glycosaminoglycans such as heparin, heparan, or chondroitin. The level of solubility of compounds of the 15 invention is readily available to one skilled in the art, *e.g.*, via the DrugBank Database (Wishart et al. (2008) *Nucleic Acids Res*. 36(Database issue):D901-6; Wishart et al. (2006) *Nucleic Acids Res*. 34(Database issue):D668-72) and Material Safety Data Sheets. In certain embodiments, 20 the hydrophilic pharmaceutical compound is a chemotherapeutic agent. In particular embodiments, the hydrophilic pharmaceutical compound is cisplatin.

In contrast, a hydrophobic pharmaceutical compound is a compound that exhibits poor water solubility, *e.g.*, 25 solubility less than solubility about 100 μg/ml, and is compatible with a typical lamellar lipid bilayer, such that it is spontaneously incorporated or intercalated into the bilayer. Examples of hydrophobic pharmaceutical compounds include, but are not limited to, chemotherapeutic agents 30 such as paclitaxel, docetaxel, curcumin, etoposide, or vinca alkaloids; antiinflammatory agents such as indomethacin, ketoprofen, dichlofenac, piroxicam, tenoxicam, or naproxen; antifungal agents such as

itraconazole or ketoconazole; sex hormones such as testosterone, estrogen, progesterone, or estradiol; steroids such as dexamethasone, betamethasone, or triamcinolone acetonide; antihypertensive agents such as
5 ramipril; antibiotics; cyclosporines; prostaglandins; and vitamins such as tocopherols. In certain embodiments, the hydrophobic pharmaceutical compound is a chemotherapeutic agent. In particular embodiments, the hydrophobic pharmaceutical compound is curcumin.

10 For the purposes of the present invention, a natural compound or product is a chemical or substance produced by a living organism. A natural compound is found in nature and in accordance with this invention has a pharmacological or biological activity. As is conventional in the art, a
15 natural product can be considered as such even if it can be prepared by total synthesis. Examples of natural compounds include, but are not limited to morphine; quinine; paclitaxel; vinca alkaloids such as vincristine, vinblastine, vinorelbine, and vindesine;
20 epipodophyllotoxins such as etoposide or tertiposide; cephalosporins; tetracyclines; aminoglycosides; rifamycins; chloramphenicol; lovastatin; ciclosporin; curacin A; eleutherobin; discodermolide; bryostatins; dolostatins; curcuminoids such as curcumin; cephalostatins; actinomycins
25 such as dactinomycin (actinomycin D); hormones such as testosterone, estrogen, progesterone; anthracyclines such as daunorubicin (daunomycin, rubidomycin) and doxorubicin; bleomycins such as bleomycin $A_2$ and $B_2$; plicamycin (mithramycin); mitomycin (mitomycin C); L-asparaginase;
30 interleukins such as IL-12 or IL-9; interferons such as IFNγ, IFN-α, or IFN-β; haemoglobin; antibodies; nucleic acid molecules; and glycosaminoglycans such as heparin,

heparan, or chondroitin. In particular embodiments, the natural compound is curcumin.

Synthetic, including semi-synthetic, compounds are compounds designed and synthesized in a laboratory.
5 Synthetic compounds include, but are not limited to docetaxel, imatinib mesylate, cisplatin, dexamethasone, itraconazole and synthetic analogs of natural compounds. In particular embodiments, the synthetic compound is cisplatin.

10 In accordance with particular embodiments of the invention, the hydrophilic pharmaceutical compound can be a synthetic compound or natural compound. Likewise, the hydrophobic pharmaceutical compound can be a synthetic compound or natural compound. Thus, in one specific
15 embodiment, at least one synthetic hydrophilic pharmaceutical compound is used in combination with at least one natural hydrophobic pharmaceutical compound. In another specific embodiment, at least one natural hydrophilic pharmaceutical compound is used in combination
20 with at least one synthetic hydrophobic pharmaceutical compound. In a further specific embodiment, at least one or natural hydrophilic pharmaceutical compound is used in combination with at least one natural hydrophobic pharmaceutical compound. In particular, the present
25 invention embraces a liposome formulation having at least one hydrophilic chemotherapeutic agent loaded in the aqueous core of the liposome and at least one hydrophobic chemotherapeutic agent in the bilayer of the liposome.

As demonstrated herein, the co-encapsulation of two
30 chemotherapeutic agents provided an enhancement in anti-tumor activity and prevented the development of resistance. Accordingly, it contemplated that the co-encapsulation of multiple pharmaceutical compounds will provide an

enhancement in the activity of one or more of the compounds co-encapsulated, and/or provide synergistic or complementary effects. In this regard, U.S. Patent No. 6,787,132, incorporated herein by reference, describes a variety of cytokines useful for enhancing the anti-tumor activity of chemotherapeutic agents.

Thus, in accordance with the experimental results presented herein, particular embodiments of the present invention also embrace a method for preventing the development of chemotherapeutic drug resistance by administering to a subject in need of treatment, *e.g.*, a subject with cancer, an effective amount of a liposome formulation containing at least two chemotherapeutic agents. By using the liposomal formulation of the present invention, it is possible to reduce the tendency of cancer cells subjected to chemotherapy to develop resistance to the chemotherapeutic agents used for chemotherapy. As such, the cancer can be more effectively treated. As is conventional in the art of liposome-mediated delivery of therapeutic agents, the instant liposome formulation can be administered intravenously, intraperitoneally, to an isolated portion of a mammalian body particularly a human body, such as an arm or leg, or in the case of a human, a hand, or can be injected directly into a tumor.

The present invention provides a single liposomal carrier for delivering a synthetic compound (*e.g.*, Cisplatin, doxorubicin, or gemcitabine) in the aqueous core with a natural anticancer compound (*e.g.*, curcumin, amooranin, etc.) in the liposomal bilayer. Advantageously, the natural compound serves to enhance the activity and increase the availability of synthetic compound. In so far as the synthetic compound is sequestered inside the aqueous

core of the liposome, side-effects associated with the synthetic compound are highly mitigated.

The invention is described in greater detail by the following non-limiting examples.

**Example 1: Materials and Methods**

*Liposome Preparation.* Lipids Dimyristoyl Phosphatidylcholine (DMPC) and Dimyristoyl Phosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Curcumin and Cisplatin (cis-Diammineplatinum (II) dichloride) were purchased from Sigma Aldrich (St. Louis, MO). Squamous oral cancer cells lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in DMEM:F12 with 10% fetal bovine serum and antibiotics. Liposomes were prepared using DMPC:DMPG in the molar ratio 1:1. The lipid film hydration method was used to test different lipid to drug ratios (10, 20, 50, 70 wt/wt) to determine the optimal encapsulation efficiency.

Curcumin and lipids were dried together under nitrogen gas. The film was further dried under vacuum to remove traces of the organic solvent. The curcumin-lipid film was then hydrated with an aqueous solution of cisplatin dissolved in 0.9% sterile saline with vigorous agitation to form multi-lamellar liposomes (MLVs). The MLVs were centrifuged at 10000*g for 15 minutes to purify the liposomes from the unencapsulated drug components (Mohammed, et al. (2004) *Internat. J. Pharmaceut.* 285:23-34).

To form small unilamellar vesicles (SUVs), the multi-lamellar liposomes were extruded through polycarbonate membranes of pore sizes 0.2 $\mu$m. The unencapsulated curcumin

and cisplatin were removed using SEPHADEX G-50 macrospin columns.

Large unilamellar vesicles (LUVs) were formed using the reverse phase evaporation method (Szoka, et al. (1978) *Proc. Natl. Acad. Sci. USA* 75(9):4194-4198). Lipids and curcumin were dissolved in 3 ml of diethyl ether. To increase the solubility of lipids, a small amount of methanol (~ 250 μl) was added to the diethyl ether. One ml of aqueous phase (cisplatin dissolved in 0.9% sterile saline solution) was added to 3 ml of organic phase. The mixture was bath sonicated at a temperature below 10°C until a homogenous one-phase system was obtained. The diethyl ether was then evaporated under slow rotation and reduced pressure until a viscous gel was formed. Addition of aqueous phase to the viscous gel resulted in formation of large unilamellar vesicles.

*Encapsulation Efficiency*. The encapsulation efficiency (EE) was determined after purification of the vesicles from the unencapsulated drug. The unencapsulated drug was separated from the liposomes using either a conventional centrifugation method or column chromatography method. One hundred μl aliquots of the parent and the purified samples were disrupted using methanol. The amount of curcumin in both the samples was assayed spectrophotometrically at 427 nm (Began, et al. (1999) *J. Agric. Food Chem.* 47(12):4992-4997). Cisplatin was quantified using Inductively coupled Mass Spectroscopy (ICP-MS) (Huang, et al. (2006) *J. Pharmaceut. Biomed. Anal.* 40(2):227-234). The percent encapsulation efficiency was calculated as: EE (%) = (Drug content in purified sample/Drug content in parent sample) * 100.

*Liposomal Size Determination*. The sizes of the different types of vesicles were determined via photon

correlation spectroscopy (PCS), using a NICOMP ZLS Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). The sample preparation for the DLS experiments was performed using guidelines described in the art (Frantzen, et al. 5 (2003) *AAPS PharmSciTech* 4:E36). The PCS instrument used was a NICOMP 380 with a fixed 90° scattering and external fiber angle, and a 632.8 nm helium-neon laser. The vesicles were suitably diluted using distilled water in order to achieve counts between 250 - 300 KHz. Three independent 10 samples were taken, which were measured at least 3 times.

*Transmission Electron Microscopy*. Transmission Electron Microscopy (Philips, 200 kV) and Confocal Laser Scanning Microscopy were employed to ascertain the size and morphology of curcumin loaded liposomes. Carbon coated 15 grids were deposited with the sample, which was then negatively stained with 2% ammonium molybdate.

*In Vitro Release Analysis*. *In vitro* release studies were used to analyze the leakage of curcumin and cisplatin from MLVs and SUVs. Liposomal solutions were adequately 20 diluted and aliquots were incubated at 37°C. At preselected intervals, the aliquots were withdrawn and the released drug was separated using column chromatography. The curcumin remaining within the liposomes was then quantified spectrophotometrically at 427 nm, while the cisplatin was 25 quantified using ICP-MS. For study of curcumin and cisplatin leakage from multilamellar vesicles, at chosen time intervals, the aliquots were centrifuged at 10000xg for 15 minutes and the released drug in the supernatant was quantified as described above. The percent leakage was 30 calculated as: % Drug Leakage = $EE\%_{(0\ hr)} - EE\%_{(t\ hr)}$.

*In vitro Stability Analysis*. The different liposomal types were stored at 4°C and the amount of drug leaked out from the liposomes was quantified over a period of 4 weeks

to assess the stability of the vesicles. To quantify leakage, the different liposomal types were monitored every week and the encapsulation efficiency was compared to day one.

*In Vitro Cytotoxicity Analysis*. *In vitro* cytotoxicity studies were used to determine whether liposome-encapsulated compounds were delivered more effectively and whether the compounds exhibited a synergistic effect. Approximately 5000 cells were seeded in 96 well plates and were allowed to attach and proliferate for 24 hours. In addition to free curcumin and cisplatin, various combinations of test compounds were added at several concentrations and the cells were incubated for 24 hours. In addition, different Curcumin-Cisplatin loaded liposomes were added to the cells and incubated for 24 hours. The cells were then rinsed with PBS and 20 $\mu$l of MTT solution was added to each well. The plate was incubated in the dark for formation of formazan crystals, which were then dissolved in 100 $\mu$l of DMSO. The plate was spectrophotometrically read at 570 nm. The cells were then incubated with increasing concentrations of the different liposomes: MLVs, SUVs and LUVs. The cells were treated with the drug containing liposomes for a period of 24 hours.

*Inhibition of Cisplatin-Resistance*. SCC9 cells were cultured in 96-well plates. Three treatment groups were analyzed: Cisplatin SUVs, Curcumin SUVs and Cisplatin-Curcumin SUVs. The cells were targeted with progressively increasing dosages of nanoliposomes to induce resistance. Four different starting dosages of Cisplatin were employed; 100 nM, 250 nM, 500 nM and 1 $\mu$M. The cells were rinsed and seeded in fresh culture media after every 24-hour incubation with the drug loaded nanoliposomes. At the end

of the 15 days, the viability of all the groups was assessed using the MTT assay.

*Statistical Analysis.* One-way ANOVA was used to perform statistical analyses on the different treatment groups. Tukeys comparison test was used to test the different means. An alpha level of 0.05 was set up as the significance level.

**Example 2: Physical Characterization**

Liposomes were prepared with different techniques to obtain multi-lamellar vesicles (MLV), small uni-lamellar vesicles (SUV) and large uni-lamellar vesicles (LUV). Curcumin and cisplatin were co-loaded into DMPC:DMPG liposomes at ratios of 1:10 and 1:50 respectively, with respect to the amount of lipid. The percent loading of curcumin (into the bilayer) and cisplatin (in the aqueous core) in the different liposomal systems is presented in Table 1.

TABLE 1

| Liposomal Type | Percent Encapsulation | | Particle Size (μm) |
|---|---|---|---|
| | Curcumin | Cisplatin | |
| MLV | 84.21 ± 4.22 | 11.17 ± 1.34 | 1.579 ± 0.203 |
| LUV | 65.69 ± 3.92 | 47.27 ± 1.24 | 0.872 ± 0.196 |
| SUV | 80.05 ± 1.56 | 4.94 ± 1.45 | 0.125 ± 0.004 |

When curcumin was incorporated into the bilayer, the MLVs incorporated the highest amount of curcumin followed by SUVs and LUVs ($p < 0.05$). With respect to cisplatin, the LUVs had a large internal aqueous volume and incorporated about 47% of the starting drug concentration. Since the SUVs had the smallest capture volume, the percent encapsulation of cisplatin was very low, almost <5%. The MLVs incorporated about 10% of this hydrophilic drug. The

encapsulation efficiencies for cisplatin were significantly different for the three types of liposomes ($p<0.05$).

Size characterization of the liposomes was carried out via transmission electron microscopy (TEM) and confocal microscopy. This analysis indicated that the multi-lamellar liposomes were formed in a range of sizes starting from 0.5 μM to 6 μM. TEM analysis of extruded small uni-lamellar liposomes indicated a size of approximately 150 nM. The multi-lamellar liposomes were passed through polycarbonate filters of the size 0.2 μM, and dynamic light scattering (DLS) indicated that the size of SUVs was approximately 125 nM (see Table 1). For the multilamellar liposomes, the size estimation with the DLS was about 1.5 μM. Thus, there was a good correlation between the two distinctly different methods of size characterization of the liposomal systems.

**Example 3: *In Vitro* Release Analyses**

To gain insight into the release profile of drug-loaded liposomes once injected into the body, *in vitro* release analysis of curcumin-loaded liposomes was conducted at 37°degrees. The release of curcumin followed an upward trend for all the three liposomal systems, with LUVs leaking large amounts of the drug. The release from MLVs and the SUVs was not significantly different, peaking at about 25% by the end of 6 hours. The LUVs, on the other hand, leaked almost 40% of the drug from the membrane. At the end of 24 hours, some of the extraliposomal drug seemed to reassociate with the membrane causing a drop in the release amounts. Since the lipids in the formulation, DMPC and DMPG, have transition temperatures around 23 and 25°C, respectively, the bilayers would be expected to be fairly fluid at 37°C. Hence, the leakage of the curcumin, which

was incorporated within the bilayer, would be easily facilitated.

The release of cisplatin from the different types of liposomes was dependent on the size of the carrier. The multi-lamellar systems, being the largest, leaked almost 90% of the drug at the end of 24 hours, with an initial burst release of 80% within the first two hours. Similarly, the LUVs leak about 60% of the drug at the end of 2 hours and the release plateaued to about 80% by 24 hours. The small unilamellar vesicles were the most stable leaking only 20% of the drug in the initial 2 hours and slowly increasing up to 60% at the end of 24 hours. The cisplatin within the aqueous core was also released due to the increased fluidity of the liposomal membrane. For the LUVs, the curcumin was almost completely retained within the liposomes.

**Example 4: *In Vitro* Stability Analyses**

Drug retention within liposomes is an important parameter to be considered before large scale manufacture of drug delivery systems. Early breakdown of liposomal carriers resulting in leakage of the majority of the drug upon storage can depreciate the value of the carrier, despite it possessing good treatment benefits. Accordingly, encapsulation efficiency of the two different drugs loaded within DMPC:DMPG liposomes was monitored every week for a total of 4 weeks for SUV, LUV, and MLV liposomes stored at 4°C. The results of this analysis indicated that more than 70-80% of cisplatin was retained within the three types of liposomes at the end of 4 weeks. For curcumin, almost 50% of the initial amount of curcumin loaded remained within the bilayer of SUV, LUV, and MLV liposomes at 4°C. These results indicated that the DMPC:DMPG liposomes had a very

good shelf-life and could be stored in the lyophilized condition for future reconstitution.

**Example 5: *In Vitro* Cytotoxicity Analyses**

5 The effect of curcumin-cisplatin co-loading on squamous oral cancer cells was also investigated. Different treatment groups were analyzed and the MTT assay was used to quantify the cell viability at a range of dosing levels. For Cisplatin, the $IC_{50}$ was 12.41 $\mu$m, while the $IC_{50}$ for 10 curcumin was 40.81 $\mu$m. The cells were also treated with the combination of curcumin:cisplatin at a ratio of 4:1, resulting in an $IC_{50}$ at curcumin dose of 30.76 $\mu$M and cisplatin dose of 7.69 $\mu$M.

The response of the cells to the same drugs in the 15 liposomal form was then assessed by incubating the cells with the three liposomal systems, MLVs, SUVs and LUVs. This analysis showed a greater response was achieved with the LUVs ($IC_{50}$ of 25 $\mu$M) as the loading of cisplatin was significantly higher than MLVs and SUVs. For SUVs, the dose 20 response was lower than that with the LUVs and MLVs with an $IC_{50}$ of 35 $\mu$M, as the loading of both the drugs was significantly lower. With the MLVs, the $IC_{50}$ achieved was 27 $\mu$M, which was not significantly different that that of LUVs ($p < 0.05$).

25 The efficacy of the co-loading in bringing down the $IC_{50}$ was examined by comparing the treatment effects of single drug and multidrug loaded liposomes. The dose response curves indicated $IC_{50}$ values for curcumin and cisplatin liposomes of 32 $\mu$M and 16.77 $\mu$M, respectively. As 30 shown in Figure 1, the induced cytotoxic effects of co-loaded liposomes were significantly higher than that of curcumin liposomes alone ($p < 0.05$).

To determine whether co-loading inhibited the development of cisplatin resistance in SCC9 cancer cells, cisplatin sensitive SCC9 cells were seeded in 96-well plates and subjected to three different treatment groups; curcumin, cisplatin and multi-drug (*i.e.*, curcumin and cisplatin) liposomes. The cells were challenged with progressively increasing dosages of each liposome. Four different starting dosages were used, 100 nM, 250 nM, 500 nM and 1 μM. At the end of 15 days, the viability of the cells in each group was tested using the MTT assay. For the starting dose of 100 nM, the percent viability of cells treated with multi-drug liposomes was 64%, which was not significantly different from the other two groups (Figure 2). However, for doses of 250 nM, the percent viability upon incubation with multi-drug liposomes was 25% as compared to 45% and 68% in the cisplatin and curcumin SUVs treatment group, respectively. In case of 500 nM and 1 μM, the viability with the multi-drug liposomes after 15 days was less than 1%, which was significantly lower than 21% and 15% of the cisplatin and curcumin groups.

To further analyze the development of drug resistance, cell viability profiles were generated for cells exposed to multi-drug liposomes as well as cisplatin and curcumin liposomes alone at starting doses of 500 nM and 1 μM. As depicted in Figure 3, the results of this analysis indicated that as the treatment progressed (*e.g.*, at the end of the 15th day) for starting doses of 500 and 1000 nM, almost 50% and 20% of the cells survived for the single-drug treatments. This implied that when the cells were treated with single-drug liposomes, they developed some resistance to the progressively increasing doses. In stark contrast, less than 1% of the cells survived to day 15 in the multi-drug liposome treated groups as compared to 32%

on day 7. These values demonstrate that the presence of drugs in combination in the liposomes prevented the development of resistance to any single drug in the combination.

**Example 6: *In Vivo* Analyses**

Efficacy of multi-drug-loaded liposomes containing at least one chemotherapeutic agent can be determined based upon the survival rate of BALB/c mice infected with tumor cells. Any suitable animal model of cancer (including mouse, rat or primate) can be employed. By way of illustration, the instant formulations can be analyzed in lung and subcutaneous colon carcinoma models of cancer.

*Lung Adenocarcinoma Model*. Several groups of BALB/c mice are injected intraperitoneally with 5 X $10^5$ M109 tumor cells (day 0). Multi-drug-loaded liposomes containing at least one chemotherapeutic agent in the lipid bilayer and at least one chemotherapeutic agent in the aqueous core are given once daily on, *e.g.*, days 10, 13 and 16. Control groups receive no treatment, receive free chemotherapeutic agent(s), or drug-free liposomes. Each group is inspected for survival up to 100 days after tumor inoculation. The number of survivors at the end of the experiment and the median survival time are obtained. It is expected that a liposome containing at least one chemotherapeutic agent in the lipid bilayer and at least one chemotherapeutic agent in the aqueous core will be more effective than either chemotherapeutic agent alone, *i.e.*, mice receiving chemotherapeutic agents alone will show lower survival rates.

*Subcutaneous Colon Carcinoma Model*. In this test, several groups of BALB/c mice are injected in the footpad with $10^5$ C26 colon carcinoma cells. Seven days later, free

drug or multi-drug-loaded liposomes containing at least one chemotherapeutic agent in the lipid bilayer and at least one chemotherapeutic agent in the aqueous core are administered and the number of tumor free mice is determined. It is expected that a liposome containing at least one chemotherapeutic agent in the lipid bilayer and at least one chemotherapeutic agent in the aqueous core will have significantly few tumors and/or smaller tumors than in the other groups.

Based upon the analyses in one more model systems, liposomes of the invention can be used in humans. Administration can be by intraperitoneal (ip), subcutaneous (sc), intravenous (iv), intraarterial (ia), or intramuscular (im) injection. Liposomes in the form of large multilamellar vesicles (MLVs) are preferred for intraperitoneal, subcutaneous or intramuscular administration, while SUVs are preferred for intravenous as well as intramuscular administration. Specific time intervals, doses and courses of treatment can be varied depending on the extent of symptoms, the chemotherapeutic agents employed and the condition of the patient.

**What is claimed is:**

1. A liposome formulation having at least one hydrophilic pharmaceutical compound loaded in the aqueous core of the liposome and at least one hydrophobic pharmaceutical compound in the bilayer of the liposome.

2. The liposome formulation of claim 1, wherein the hydrophilic pharmaceutical compound is a synthetic compound and the hydrophobic pharmaceutical compound is a natural compound.

3. The liposome formulation of claim 1, wherein the hydrophilic pharmaceutical compound is a natural compound and the hydrophobic pharmaceutical compound is a synthetic compound.

4. The liposome formulation of claim 1, wherein the hydrophilic pharmaceutical compound and the hydrophobic pharmaceutical compound are natural compounds.

5. The liposome formulation of claim 1, wherein the hydrophilic pharmaceutical compound and the hydrophobic pharmaceutical compound are chemotherapeutic agents.

6. A method for preventing the development of chemotherapeutic drug resistance comprising administering to a subject in need of treatment an effective amount of the liposome formulation of claim 5, thereby preventing the development of chemotherapeutic drug resistance.

7. A method of producing a multi-drug-loaded liposome comprising combining

(a) a lipid and hydrophobic pharmaceutical compound mixture with

(b) an aqueous phase containing a hydrophilic pharmaceutical compound

5 thereby producing a multi-drug-loaded liposome.

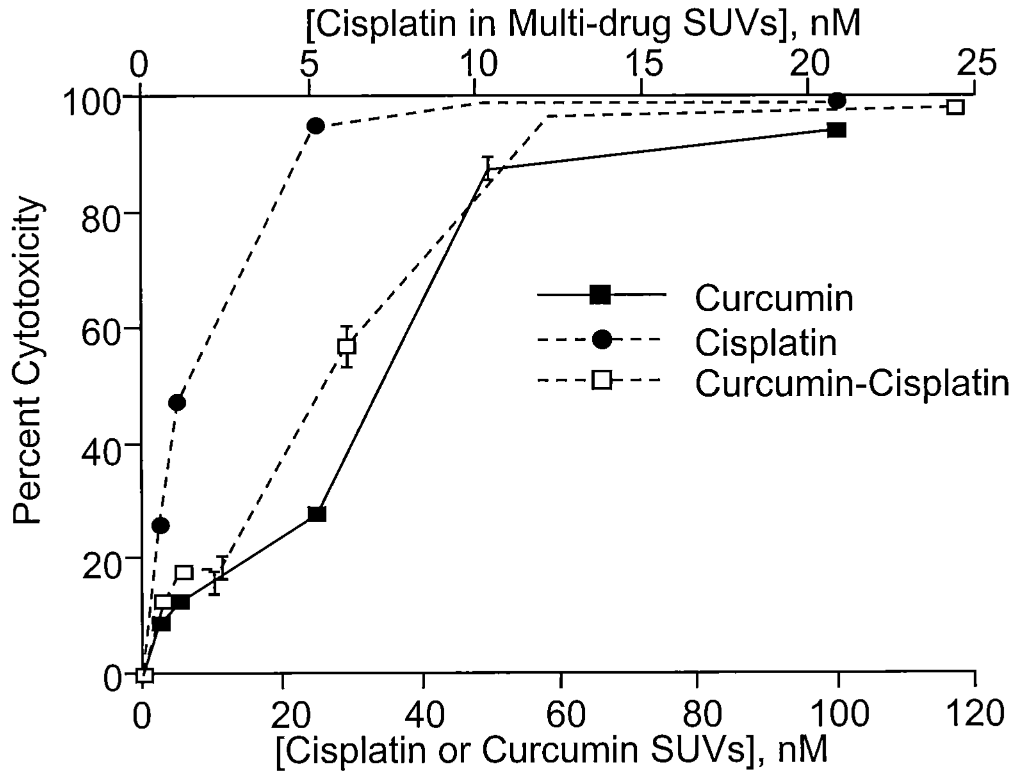


*FIG. 1*

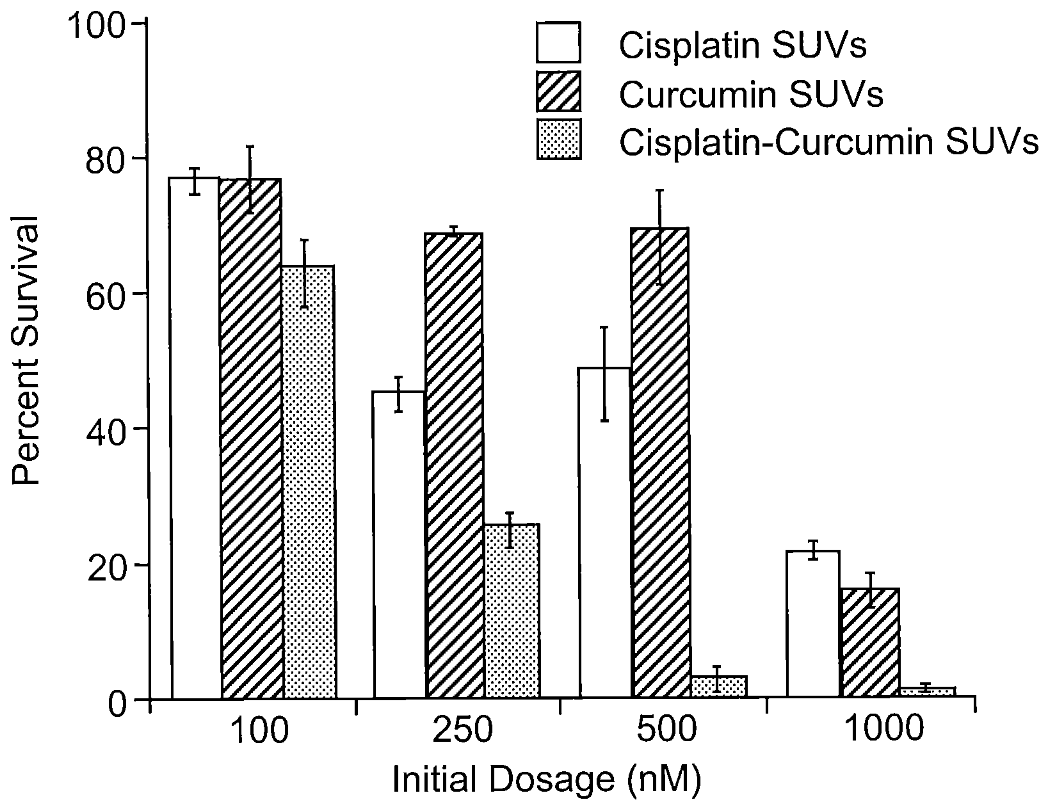


*FIG. 2*

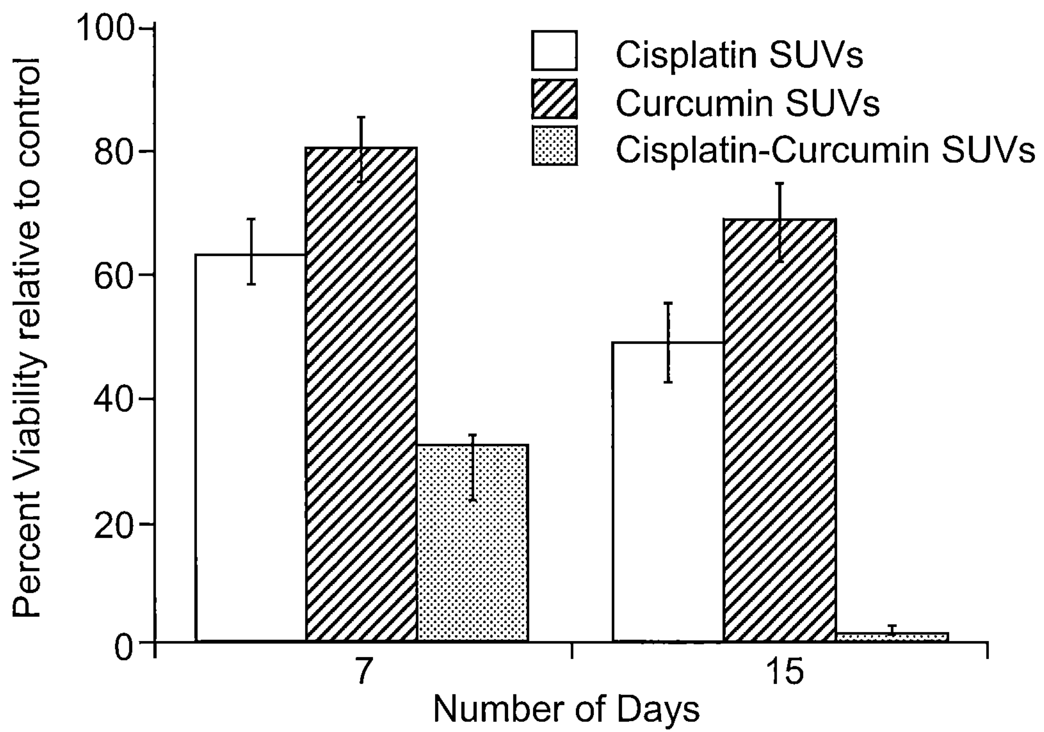


*FIG. 3A*

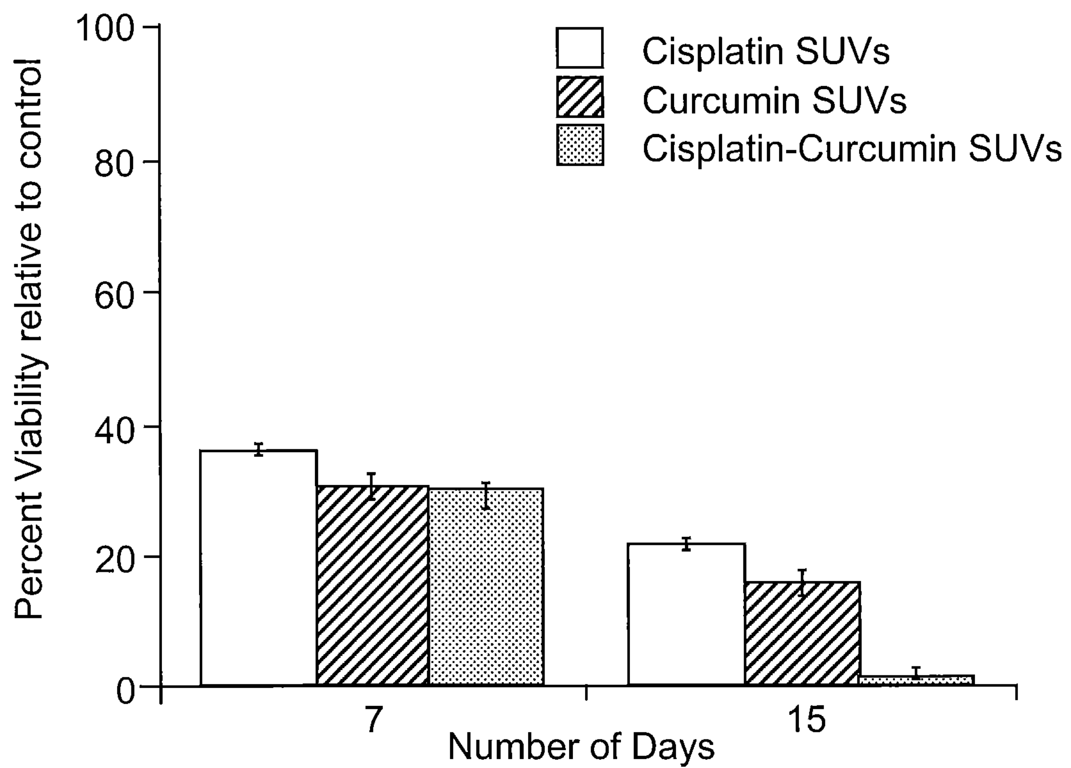


*FIG. 3B*

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/50622

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 9/127, A61K 51/00, A61M 36/14 (2009.01)
USPC - 424/1.21, 424/450
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC – 424/1.21, 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC–424/9.321, 424/9.51, 424/94.3, 424/417, 424/470 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST -- PGPB,USPT,USOC,EPAB,JPAB; Dialog Classic Files ? 654, 652, 351, 349, 315, 6, 35, 65, 155; Google Scholar; USPTO Web Page; Search terms – liposome, hydrophobic drug, hydrophilic drug, natural drug, synthetic drug, chemotherapeutic agent, chemotherapy resistance, liposome loading

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>--<br>Y | US 2008/0075762 A1 (TARDI et al.) 27 March 2008 (27.03.2008) para [0024], [0027], [0033], [0035], [0038], [0051], [0060], [0098], [0127], [0140], [0143]-[0145], Fig 3A, 4, 6, 24 | 1-5, 7<br>--------<br>6 |
| Y | US 2007/0243548 A1 (GEORGES et al.) 18 October 2007 (18.10.2007) para [0002], [0017], [0018], [0029], [0061], [0113] | 6 |

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 August 2009 (13.08.2009) | 31 AUG 2009 |

| Name and mailing address of the ISA/US | Authorized officer: |
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| Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, Virginia 22313-1450<br>Facsimile No. 571-273-3201 | Lee W. Young<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |